

IN VITRO STUDY OF PHYLLOSHERE BACTERIA AS PROMISING BIOCONTROL AGENTS AGAINST BACTERIAL LEAF BLIGHT DISEASE (*Xanthomonas oryzae* pv. *oryzae*) IN RICE PLANTS

Rachmi Masnilah^{1,3}, Fenny Nurmala², Ankardiansyah Pandu Pradana^{1,4*}

¹ Plant Protection Study Program, Agriculture Faculty, University of Jember, Jember – 68121, Indonesia

² Agrotechnology Study Program, Agriculture Faculty, University of Jember, Jember – 68121, Indonesia

³ Plant Disease Research Group, University of Jember, Jember – 68121, Indonesia

⁴ Healthy Agriculture Research Group, University of Jember, Jember – 68121, Indonesia

*E-mail Corresponding author: pandu@unej.ac.id

ABSTRACT

This study aimed to explore the potential of phyllosphere bacterial isolates as biological control agents against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). We isolated a strain exhibiting typical morphological and biochemical characteristics of *Xoo*, such as a yellowish, spherical colony, Gram-negative, and starch hydrolyzing ability. This isolate was shown to cause necrotic lesions on rice leaves, corroborating its pathogenic nature. Subsequently, we isolated ten diverse phyllosphere bacteria from rice plants. These isolates were characterized based on various morphological and biochemical attributes including colony shape, margin appearance, color, growth time, and the ability to fluoresce under UV light when cultivated on King's B medium. Crucially, none of these isolates induced a hypersensitive response in tobacco leaves, an initial indicator of their potential as safe biological control agents. In an antagonistic assay against *Xoo*, all the phyllosphere isolates demonstrated varying levels of inhibition, suggesting their potential role in biological control. Two isolates, PGM1 and PGM4, exhibited the highest antagonistic effects against *Xoo*. These findings provide preliminary evidence for the potential use of these phyllosphere isolates in managing bacterial leaf blight in rice, warranting further research to confirm their efficacy and safety under field conditions.

Key words: antagonism, biocontrol, bacteriostatic, phyllosphere, *Xoo*

INTRODUCTION

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is a pathovar of bacteria known for causing bacterial blight, a serious disease that mostly affects rice, a staple food for a large section of the world's population [1]. Maintaining the health of rice crops is critical to ensuring food security [2]. Bacterial blight is most common in Asia, which is a major rice-growing region. However, it is important to highlight that the consequences of this disease spread beyond the borders of this specific location. This pathovar has been found in both temperate and tropical settings, proving its adaptability and vast geographic range [3]. Bacterial blight is a serious concern in rice farming because of its capacity to generate large outbreaks [4].

Infected rice plants can suffer significant agricultural production losses, with estimated yield losses ranging from 20.6% to 35.8% during the wet season and 17.5% to 28.0% during the dry season [5]. Stunted plant growth, poor grain development, decreased grain quality, and a high incidence of broken rice are typical indications of *Xoo* infection in rice plants [1] [4]. Leaf blight and withering are visible signs. Wilting mostly affects young plants and normally appears between one and six weeks following the first sowing. Wet streaks or lesions are seen along the leaf edges at the start of the illness. The detected lesions have a growing tendency and a pigmentation shift to grayish-green, resulting in leaf wrinkling. The afflicted leaf ultimately wilts and resembles being scalded with hot water [6]. Withering is a sign of bacterial leaf blight and is considered severely destructive. The second symptom, leaf blight, usually appears in fully established plants. The disease begins with the formation of wet lesions on one or both leaf surfaces, usually a few centimetres from the leaf tip. The detected lesions enlarge, with a grayish-green color and a wet

texture, eventually leading in the creation of crinkled and dehydrated leaves with a whitish-gray pigmentation. Initially, the lesions seem like water-soaked streaks along the leaf borders [7].

Despite repeated efforts, *Xoo* management in rice fields remains a serious challenge. The growing public understanding of the significance of eating nutritious foods has resulted in a stronger emphasis on long-term and health-focused *Xoo* management measures [8]. Among the emerging approaches, biological control agents have emerged as a potential strategy. Understanding the bioecology of *Xoo* shows that using biological control agents that occupy a comparable ecological niche may produce better results [9]. Living creatures or naturally occurring compounds that can successfully suppress the population and activity of *Xoo* are referred to as biological control agents. This technology uses natural mechanisms to provide an eco-friendly and long-lasting alternative to standard chemical-based treatments [10] [11].

In light of the above information, it is crucial to emphasize that among the great array of microorganisms present on plants, phyllosphere bacteria stand out as a prominent category. These bacteria, which number in the thousands, serve an important role in preserving ecological balance and fostering plant health [12]. As one of their key tasks, they act as biological control agents, successfully controlling numerous plant diseases such as *Xoo*. Because Phyllosphere bacteria have the same biological niche as *Xoo*, they can have a substantial impact on bacterial blight control and prevention. The ability of these organisms to act as innate enemies, vying with *Xoo* for limited resources and epidermal area on the leaf, is an optimistic indicator for reducing the development and spread of this damaging disease [13]. Furthermore, the presence of phyllosphere bacteria has the ability to stimulate the plant's natural defense systems, hence increasing resistance to *Xoo* and other diseases [14].

Bacteria in the phyllosphere are known to influence *Xoo* through a number of methods. For starters, these organisms can synthesize antimicrobial chemicals like siderophores, which can bind and sequester the critical iron ions necessary for *Xoo* survival and multiplication [15]. This method deprives the pathogen of crucial nutrients, impairing its capacity to grow. Furthermore, bacteria in the phyllosphere compete directly with *Xoo*, competing for scarce nutrients and occupying space on the leaf surface, restricting its multiplication. Finally, particular strains of phyllosphere bacteria can activate the plant's intrinsic defense mechanisms against *Xoo*, such as phytoalexin production or an improved antioxidant response [16].

Through a variety of ways, phyllosphere bacteria play an important role in the biological control of *Xoo*, therefore considerably contributing to the maintenance of rice plant production and health. The ability to limit the growth and spread of *Xoo* via natural mechanisms is a practical and ecologically benign technique for the management of bacterial blight and the maintenance of rice farming [17]. Continued investigation into the precise interactions and efficiency of diverse phyllosphere bacteria has the potential to lead to the creation of innovative techniques for battling this infection, ultimately boosting global food security [18].

Phyllosphere bacterial taxa that have shown efficiency against *Xoo* include *Pseudomonas*, *Bacillus*, and *Streptomyces*. *Pseudomonas fluorescens* and *Bacillus subtilis* have been shown to be effective against a range of plant diseases, including *Xoo* [19] [20]. Enzymatic processes are used by a considerable fraction of phyllosphere bacteria to regulate and suppress pathogen development. The bacteria *Pseudomonas fluorescens* is known to generate hydrolytic enzymes such as proteases and lipases. These enzymes are capable of damaging *Xoo*'s cell walls, preventing it from growing.

Furthermore, both *Pseudomonas* and *Bacillus* are capable of producing enzymes that can damage the cellular architecture of other species, such as chitinases and -1,3-glucanases [21]. This feature significantly boosts their effectiveness against *Xoo*. Given the shown potential of these phyllosphere-dwelling bacteria, it is critical to do studies specifically aimed at assessing their capacity to regulate *Xoo*.

MATERIALS AND METHODS

Pathogen Isolation

Xoo was isolated by collecting leaf samples from rice plants that had been infected with *Xoo* in the field. After washing the leaves with sterile water, 3 grams of the sample were ground with sterile water. The resultant suspension was then serially diluted up to 10^{-5} . The suspension was then cultivated on nutrient agar (NA) (HiMedia, India) and incubated for 48 hours. Colonies of *Xoo* were discovered after the incubation period. The detected bacterial colonies were then transferred to new petri dishes to create pure cultures. The isolated bacteria were then preserved for further characterisation and testing [22].

Characterization of *Xoo*

Gram test

A little quantity of *Xoo* bacteria was deposited on a microscope slide using a loop. The mixture was then gently flooded with a 3% KOH solution and well mixed. Slowly, the loop or needle was lifted. The bacteria were Gram-negative if the isolate was lifted along with the loop. If, on the other hand, the bacteria did not attach and stayed on the slide, it indicated that they were Gram-positive [23].

Hypersensitive Reaction Test

To conduct the hypersensitive reaction test on tobacco leaves, an inoculum from the *Xoo* isolate with a density of 1×10^8 cfu ml⁻¹ was infiltrated into tobacco leaves. After that, the leaves were incubated for 48 hours. If the tobacco leaves showed necrotic signs, the tested isolate was a pathogenic bacteria [24].

Starch Hydrolysis Test

This test was carried out by taking a cultivated 24-hour-old *Xoo* bacterium isolate and incubating it for 5 days. Observations were conducted after 5 days of incubation, and the media were infected with starch reagent. A positive reaction was demonstrated by a distinct color change around the bacterial colony, whereas a negative reaction was indicated by the absence of a color change [25].

Phylosphere Bacteria Isolation

Rice plant leaves were used to extract phyllosphere bacteria. Healthy leaves were chosen by cutting intact leaf sections and washing them three times in sterile water. After that, the rice leaf segments were laid on tissue paper to dry. The leaf segments were crushed after drying, and repeated dilutions were made until a dilution of 10^{-6} was reached. The diluted samples were subsequently grown on NA (HiMedia, India) and King's B (HiMedia, India) medium for 24-48 hours at room temperature. The newly formed colonies were then cleansed by growing them on fresh media and kept for further characterisation and testing [48].

Characterization of Phylosphere Bacteria

KOH Gram Test

A loopful of a 48-hour-old bacterial isolate was put on a microscope slide, followed by a drop of 3% KOH. The loop was gently swirled into the liquid and slowly lifted. When raising the bacterial colony, the presence of mucus indicated a Gram-negative result, whereas the lack of mucus suggested a Gram-positive result [23].

Hypersensitivity Test on Tobacco

The test was carried out by infiltrating a 48-hour-incubated bacterial isolate suspension onto tobacco leaves. If necrotic signs were found on the tobacco leaves after 48 hours, the isolate was categorized as a pathogen, and vice versa [24].

Starch Hydrolysis Test

The test was carried out by cultivating a 24-hour-old bacterial isolate on starch medium and incubating it for five days. The medium was treated with starch reagent after five days of incubation. If the region around the bacterial colony became transparent, the reaction was positive; if the area around the bacterial colony became black or dark blue, the reaction was negative [25].

Growth at 45°C in Nutrient Broth (NB) Medium

Phyllosphere bacterial isolates were cultivated in NB (HiMedia, India) media and incubated anaerobically for 48 hours in sterile mineral oil at 45°C. Bacteria were grown on the same medium but incubated for 48 hours at 24°C as a control [26].

Growth at pH 5.7

Bacterial isolates were cultured for 48 hours in NB (HiMedia, India) medium adjusted to pH 5.7 and kept at 24°C for five days. The increase in turbidity in the medium suggested a favorable response [26].

Growth at 7% NaCl

The 48-hour-old bacteria were inoculated into NB (HiMedia, India) medium with 7% NaCl (Merck, Germany). For five days, the medium injected with bacteria was incubated at 24°C. The increase in turbidity in the medium suggested a favorable response [27].

Acid Reaction Test

Carbohydrate (glucose, sucrose, and mannitol at 1% concentration) were sterilized individually. The carbohydrates were then added to separate test tubes. After that, phyllosphere bacterial isolates were injected into each carbohydrate-containing media. To a depth of one centimeter, the tubes were sealed with sterile mineral oil. The tubes were then incubated at room temperature for seven days. When an acid reaction occurred, the color of the medium changed from green to yellow [28].

Potato Decay Test

Potato slices 7-8 mm thick were disinfected with 70% ethanol and washed three times with sterile water. Phyllosphere bacterial isolates were inoculated onto potato slices in petri plates coated with filter paper. Following that, the petri dishes were incubated for 24 hours at 24°C. The absence of deterioration in the potato slices injected with bacteria showed a good reaction [29].

Arginine Dehydrolase Test

The arginine dehydrolase test is used to detect bacterial growth in an anaerobic medium containing arginine. An arginine medium with phenol red as a pH indicator was utilized. In two independent test tubes, phyllosphere bacterial isolates were cultured in media containing L-arginine, one sealed with paraffin oil and the other without. The testing tube was then filled with a

24-hour-old bacterial culture. The tubes were incubated at a temperature of 28°C for four days. A favorable reaction was indicated by the hue changing to red [30].

Antagonistic Bioassay

The dual planting approach was used to test the antagonistic activity of phyllosphere bacterial isolates against *Xoo*. The isolates were inoculated with a sterile needle and cultured on NA (HiMedia, India) medium. After that, the plates were incubated at room temperature for 48 hours. Following that, the petri dishes were inverted, and a 1 mL chloroform solution was applied to the lid at the edge of each dish. The plates were restored to their original place after a 2-hour incubation. Following that, a 0.2 ml solution of *Xoo* in 0.6% agar was placed onto the plates and uniformly dispersed. After that, the plates were incubated for 24 hours to look for any inhibitory zones that had developed [31].

The clear zone around the bacterium can be sliced and transferred into peptone medium to ascertain the mechanism type of phyllosphere bacteria. After that, the medium was incubated for three days. If the medium gets hazy, the bacteria have a bacteriostatic mechanism. If the peptone medium remains clear, this indicates that the bacteria have a bactericidal mechanism [32].

RESULTS AND DISCUSSION

***Xanthomonas oryzae* pv. *oryzae* isolate**

The pathogenic bacterial isolation results showed the presence of bacterial colonies with features comparable to *Xoo*. The isolated colonies were golden in color, spherical with a smooth surface, and had even margins. These visual findings, as shown in Figure 1a, give preliminary evidence of a relationship between the isolated bacteria and *Xoo*. Further analysis of the isolates showed additional properties that correspond to *Xoo*'s recognized characteristics. Gram test investigation, as shown in Figure 1b, indicated that the bacteria were Gram-negative. The isolates also exhibited pathogenic characteristics and the ability to hydrolyze starch, as evidenced by their growth on particular medium. These data provide credence to the identification of the isolates as possible *Xoo* strains.

A hypersensitive response test was carried out to assess the pathogenicity of the acquired isolates. The test involves exposing plant tissues to the isolates and observing the necrotic signs that resulted. Figure 1c shows the appearance of necrotic lesions after inoculation of the isolates, confirming their pathogenic character. Pathogenic bacteria commonly cause the hypersensitive reaction, which is characterized by localized tissue death, in vulnerable plants. This finding adds to the evidence that the isolates have pathogenic capabilities, confirming their resemblance to *Xoo*.

Inoculation studies on rice leaves with the identified isolates provided additional proof of pathogenicity. Figure 1d shows necrotic signs on the injected leaves. This observation supports prior studies and implies that the isolates can cause disease in rice plants. The appearance of necrotic lesions on the leaves following inoculation corresponds to the typical symptoms associated with *Xoo* infection, confirming the conclusion that the isolates obtained are most likely pathogenic strains of *Xoo*.

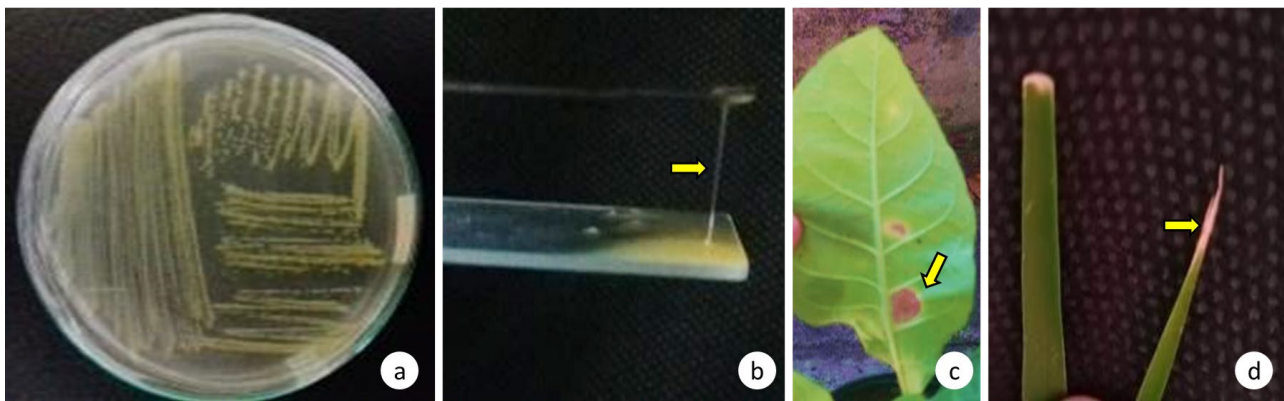


Figure 1. (a) Isolated *Xoo* colonies obtained in this study; (b) *Xoo* Gram test showing Gram-negative characteristics indicated by the presence of mucus; (c) Necrosis on tobacco plant leaves indicating a hypersensitive reaction; (d) Necrosis on rice plants demonstrating the pathogenic nature of the isolated *Xoo* strains.

Xoo colonies are distinguished by their yellow appearance, gram-negative nature, and yellow patches or lesions on the leaves of rice plants [22]. According to [33], the first indication of *Xoo* infection is the emergence of yellow spots on the plants' leaves. These marks indicate illness prevalence and act as an early warning indicator. *Xoo* isolates discovered from exploratory experiments feature yellow colonies with a spherical form, a smooth surface, and even edges. These qualities are consistent with [34], which discovered that *Xoo* colonies appear yellow due to the presence of xanthomodins, a pigment responsible for their colour.

Phyllosphere Bacterial Isolates

In this research, a successful isolation process yielded a diverse set of 10 isolates from the phyllosphere of rice plants. These isolates were carefully characterized, taking into account their unique attributes such as colony shape, margin appearance, growth time, color, and even their fluorescent capabilities when cultivated on King's B medium (Figure 2). To assess their biosafety as biological agents, a hypersensitive reaction test was conducted by inoculating the isolates onto tobacco leaves and monitoring their effects. Encouragingly, none of the ten isolates demonstrated the ability to induce necrotic symptoms on tobacco leaves within a 48-hour period. This outcome indicates that the phyllosphere bacterial isolates obtained in this study pose no harm or detrimental effects on tobacco plants, suggesting their safety and potential suitability for use as biological agents in agricultural applications. For a comprehensive understanding of the characteristics of these isolates, additional details can be found in Table 1.

Bacterial diversity in the phyllosphere is typically high, albeit not as high as in the rhizosphere. [35] discovered that effectively isolated biocontrol agents often contain phenotypic variety. Interactions between genes and the environment can cause phenotypic variations [36]. When cultivated on King's B medium, certain bacteria can fluoresce under UV light. They glow under UV light due to the formation of pyoverdine, a sort of siderophore secondary metabolite. Siderophores are iron chelating molecules with a high affinity that are released by microorganisms in low iron settings to scavenge iron from the surrounding medium. In the case of *Pseudomonas*, the pyoverdine is a fluorescent greenish pigment that can be seen when the bacteria are cultured in certain conditions such as King's B medium. By restricting the available iron, King's B medium encourages the formation of pyoverdine, causing the bacteria to create more of the siderophore in an effort to scavenge iron from the environment [37].

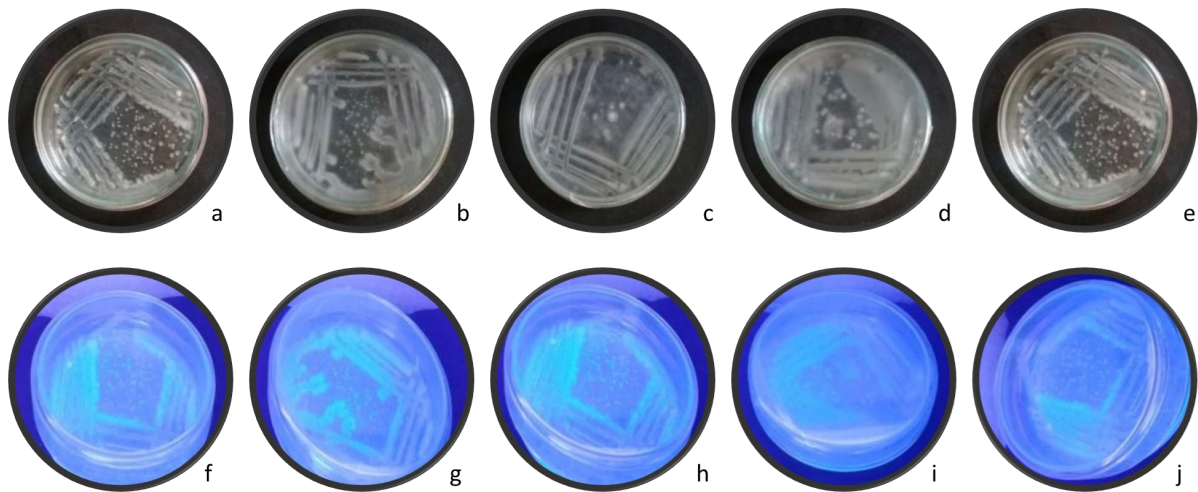


Figure 2. Phyllosphere bacterial isolates successfully obtained in this study; (a-e) sequentially showing isolates PGL2, PGL3, PGL4, PGM1, and PGM3 on NA medium; (f-j) sequentially showing isolates PGL1, PGL5, PGM2, PGM4, and PGM5 on Kings' B medium and demonstrating their florescent capability.

Table 1. Phyllosphere bacterial isolates obtained in this study and their characteristics.

Isolate Code	Characteristics	Hypersensitive Reaction
PGL1	Circular colonies with smooth edges, able to grow within 24 hours on NA medium, grayish-white in color, and fluorescent on Kings' B medium.	Negative
PGL2	Circular colonies with smooth edges, able to grow within 48 hours on NA medium, white in color, and non-fluorescent on Kings' B medium.	Negative
PGL3	Circular colonies with irregular edges, able to grow within 24 hours on NA medium, brownish-white in color, and non-fluorescent on Kings' B medium.	Negative
PGL4	Circular colonies with irregular edges, able to grow within 24 hours on NA medium, brownish-white in color, and non-fluorescent on Kings' B medium.	Negative
PGL5	Circular colonies with irregular edges, able to grow within 48 hours on NA medium, light white in color, and fluorescent on Kings' B medium.	Negative
PGM1	Circular colonies with uneven edges, able to grow within 48 hours on NA medium, reddish-white in color, and non-fluorescent on Kings' B medium.	Negative
PGM2	Circular colonies with smooth edges, able to grow within 24 hours on NA medium, greenish-white in color, and fluorescent on Kings' B medium.	Negative
PGM3	Circular colonies with uneven edges, able to grow within 48 hours on NA medium, yellowish-white in color, and non-fluorescent on Kings' B medium.	Negative
PGM4	Circular colonies with smooth edges, able to grow within 24 hours on NA medium, cloudy white in color, and fluorescent on Kings' B medium.	Negative
PGM5	Circular colonies with uneven edges, able to grow within 24 hours on NA medium, orange-white in color, and fluorescent on Kings' B medium.	Negative

When a hypersensitive reaction test was conducted on the isolates acquired in this investigation, they all showed a negative result. HR is a type of programmed cell death that occurs

in response to the immune system of a plant recognizing a specific pathogen. When a plant detects a possible pathogen, it responds by killing off the cells in the pathogen's near area, so restricting its spread [38].

If a biocontrol agent causes a hypersensitive reaction in a plant, the plant it is designed to protect might suffer considerable harm. For example, if the biocontrol agent is a bacterium that the plant perceives as a pathogen, the plant may begin a hypersensitive reaction, resulting in cell death in the vicinity of the bacteria [35]. Even though the bacteria is meant to defend the plant against other infections or pests, this might cause damage to the plant. As a result, it's critical for biocontrol agents to be hypersensitive reaction negative, which means they do not activate this defensive mechanism in the plants they are designed to protect. This guarantees that the biocontrol agent may carry out its intended activity without harming the plant [24].

Phyllosphere Bacterial Characteristics

The phyllosphere bacterial isolates characterized in this investigation displayed a variety of characters. Five isolates were Gram-positive, while the other five were Gram-negative. Testing on liquid media revealed that all isolates could grow in liquid medium at pH 5.7, with two isolates, PGL2 and PGM1, showing particularly significant growth. Growth tests with 7% NaCl yielded similar findings, as all isolates collected in this investigation were able to develop in the presence of this concentration, with just one isolate, PGM5, displaying robust growth. Isolate PGL3 failed the growth test at 45°C, showing that it was unable to develop at that temperature. The remaining isolates, on the other hand, were able to develop, with two isolates, PGL5 and PGM1, displaying particularly robust growth.

In the acid production test, all isolates reacted positively to the carbohydrates examined (glucose, sucrose, and mannitol). However, of the sucrose isolates, five (PGL1, PGL3, PGL4, PGM1, and PGM4) showed modest responses. Furthermore, all isolates were able to hydrolyze starch in the starch hydrolysis test. Similarly, all isolates showed good responses in the arginine dehydrolyase test, with the exception of isolate PGL5, which showed a mild reaction.

Characterization is critical for knowing the characteristics of bacteria in a variety of conditions. The parameters considered in this study primarily aid in the identification of bacterial species. However, it is crucial to emphasize that defining the genus or species of bacteria produced from this study based purely on measured physiological features is not adequate. However, these characteristics can provide vital insight about the bacterium's ability to survive in the phyllosphere environment.

The phyllosphere environment is inextricably linked to the surrounding environment in which the plant lives. This environmental condition changes greatly depending on the place where the plant is grown [39]. Plants living near coastlines, for example, will have a significantly higher NaCl content in their phyllosphere. Furthermore, pH and temperature within the phyllosphere tend to change depending on the type of plant, the nature of fertilizers and pesticides used, and the unique climatic circumstances in which the plant grows [40].

The efficiency of phyllospheric microorganisms in reducing phyllosphere diseases is strongly impacted by a variety of circumstances. One key feature is the microorganisms' adaptability, particularly in respect to the consumption or hydrolysis of carbohydrates and other substances. The findings also implies that bacteria capable of using a broader spectrum of carbohydrates are likely to demonstrate a higher level of adaptability, allowing for improved control efficacy [41].

Table 2. Characteristics of phyllosphere bacterial isolates obtained in this study

Characters	Isolate Code									
	PGL1	PGL2	PGL3	PGL4	PGL5	PGM1	PGM2	PGM3	PGM4	PGM5
Gram	-	+	+	+	-	+	-	+	-	-
Growth in liquid media:										
pH 5.7	+	++	+	+	+	++	+	+	+	+
NaCl 7%	+	+	+	+	+	+	+	+	+	++
Temperature 45°C	+	+	-	+	++	++	+	+	+	+
Acid formation reaction:										
Glucose	+	+	+	+	+	+	+	+	+	+
Sucrose	+*	+	+*	+*	+	+*	+	+	+*	+
Mannitol	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+
Arginine dehydrolyase	+	+	+	+	+*	+	+	+	+	+
Potato decay	-	-	-	-	-	-	-	-	-	-

Note: (+) indicates a positive result, (++) indicates a strong positive result, (*) indicates a weak positive result, and (-) indicates a negative result.

Antagonist Activity Against *Xoo*

The results of the tests revealed that all of the phyllosphere bacterial isolates identified in this investigation were capable of inhibiting *Xoo* growth in vitro (Figure 3). The inhibition values varied across the isolates, with the least being 4.3 mm and the greatest being 9.3 mm. PGL4 was the isolate with the lowest inhibition value, whereas PGM1 and PGM4 had the greatest inhibition values. The phyllosphere bacteria isolated in this study displayed a bacteriostatic inhibition mechanism, according to the analysis of the inhibitory mechanism. Table 3 also shows the inhibition levels for each tested isolate in this investigation.

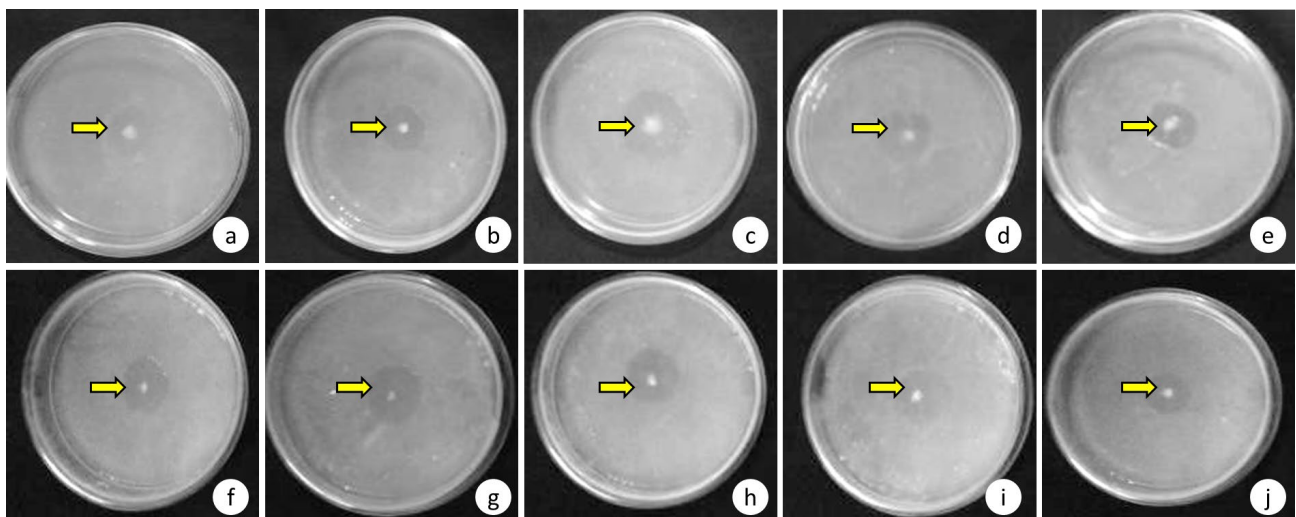


Figure 3. Inhibition of *Xoo* growth by phyllosphere bacteria; the arrow indicates the position of the clear zones formed; (a-j) in sequence represent the test results of phyllosphere bacterial isolates PGL1, PGL2, PGL3, PGL4, PGL5, PGM1, PGM2, PGM3, PGM4, and PGM5.

The effectiveness of phyllosphere bacteria in controlling *Xoo* has been highlighted in several preceding studies. The mode of action through which phyllosphere bacteria control *Xoo* can manifest in two ways - directly or indirectly [42]. In terms of direct interaction, phyllosphere bacteria can produce specific antibiotic compounds. These compounds are biologically active and perform a significant function in inhibiting, suppressing, or altogether preventing the growth and multiplication of *Xoo*. This antibiotic action interferes

with various vital processes within the pathogen, such as cell wall synthesis, protein production, or DNA replication, thereby arresting their growth and propagation [43].

Table 3. Inhibition zones and antagonistic mechanisms of phyllosphere bacterial isolates against *Xoo*.

Isolate Code	Average Inhibition Zone (mm)	Inhibition Mechanism
PGL1	5.5	Bacteriostatic
PGL2	5.6	Bacteriostatic
PGL3	8.6	Bacteriostatic
PGL4	4.3	Bacteriostatic
PGL5	7	Bacteriostatic
PGM1	9.3	Bacteriostatic
PGM2	7	Bacteriostatic
PGM3	9	Bacteriostatic
PGM4	9.3	Bacteriostatic
PGM5	5.6	Bacteriostatic

Beyond the production of antibiotic compounds, phyllosphere bacteria are also recognized for their ability to produce several specific enzymes. These enzymes can disrupt the cell wall integrity of *Xoo*, leading to cell lysis and death, thereby contributing to controlling the population of the pathogen [44]. Additionally, some phyllosphere bacteria may also outcompete *Xoo* for nutrients and space, further limiting the pathogen's ability to establish and spread within the phyllosphere environment [45].

Indirect interactions, on the other hand, involve the phyllosphere bacteria stimulating resistance mechanisms within the host plant itself. This is typically accomplished by inducing systemic resistance in plants. In this particular mechanism, the plant's defense system is primed, leading to an amplified response when the actual pathogen attack occurs [9]. For instance, the presence of beneficial phyllosphere bacteria can trigger the plants to produce more salicylic acid, a signaling molecule in plants that initiates the defense response, which can further inhibit the development of *Xoo* [45].

In light of several previous studies, some phyllosphere bacterial genera that have been reported as particularly effective in controlling *Xoo* include *Bacillus* and *Pseudomonas*. These bacteria are known for their broad-spectrum antagonistic properties [46]. *Bacillus* species are prolific producers of a range of antibiotic compounds and are often involved in induced systemic resistance. Similarly, certain species of *Pseudomonas* are recognized for their production of antibiotic compounds like phenazine and pyrrolnitrin, their capability to induce systemic resistance, and their role in promoting plant growth, thereby enhancing the overall health and resilience of the plant [47]. In summary, these phyllosphere bacteria function in tandem with the plant's innate defense mechanisms, bolstering the plant's ability to resist *Xoo* and other pathogens.

CONCLUSION

In conclusion, this study provides novel insights into the potential of phyllosphere bacteria as biological control agents against *Xoo*, a key pathogen causing bacterial leaf blight in rice. The isolated phyllosphere bacteria were characterized and demonstrated the ability to inhibit the growth of *Xoo* in vitro, marking a promising development in the search for effective biocontrol strategies. The isolates showed substantial adaptability to various conditions that mimic the

phyllosphere environment, indicating their potential survival and performance capabilities. While further studies are needed to elucidate their exact modes of action and field performance, this research represents a significant step toward sustainable and environmentally friendly disease management strategies in rice cultivation.

REFERENCES

- [1] Ji Z, Wang C & Zhao K. 2018. Rice routes of countering *Xanthomonas oryzae*. *International Journal of Molecular Sciences* 19(10): 3008.
- [2] Berry EM, Dernini S, Burlingame B, Meybeck A & Conforti P. 2015. Food security and sustainability: can one exist without the other?. *Public Health Nutrition* 18(13): 2293-2302.
- [3] Dunger G, Llontop E, Guzzo CR & Farah CS. 2016. The *Xanthomonas* type IV pilus. *Current Opinion in Microbiology* 30: 88-97.
- [4] Nakayinga R, Makumi A, Tumuhaise V & Tinzaara W. 2021. *Xanthomonas* bacteriophages: A review of their biology and biocontrol applications in agriculture. *BMC Microbiology* 21(1): 1-20.
- [5] Patil B, Jagadeesh G, Karegowda C, Naik S & Revathi R. 2017. Management of bacterial leaf blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* under field condition. *Journal of Pharmacognosy and Phytochemistry* 6(6): 244-246.
- [6] Quibod IL, Atieza-Grande G, Oreiro EG, Palmos D, Nguyen MH, Coronejo ST, Aung EE, Nugroho C, Roman-Reyna V & Burgos MR. 2020. The Green Revolution shaped the population structure of the rice pathogen *Xanthomonas oryzae* pv. *oryzae*. *The ISME Journal* 14(2): 492-505.
- [7] Arshad HMI, Naureen S, Saleem K, Ali S, Jabeen T & Babar MM. 2015. Morphological and biochemical characterization of *Xanthomonas oryzae* pv. *oryzae* isolates collected from Punjab during 2013. *Advancements in Life Sciences* 2(3): 125-130.
- [8] Jiang N, Yan J, Liang Y, Shi Y, He Z, Wu Y, Zeng Q, Liu X & Peng J. 2020. Resistance genes and their interactions with bacterial blight/leaf streak pathogens (*Xanthomonas oryzae*) in rice (*Oryza sativa* L.)—an updated review. *Rice* 13(1): 1-12.
- [9] Krishanti N, Wahyudi A & Nawangsih AA. 2015. Non-pathogenic phyllosphere bacteria producing bioactive compounds as biological control of *Xanthomonas oryzae* pv *oryzae*. *International Journal of Pharma and Bio Sciences* 6(1): 7-15.
- [10] Saleem M, Meckes N, Pervaiz ZH & Traw MB. 2017. Microbial interactions in the phyllosphere increase plant performance under herbivore biotic stress. *Frontiers in Microbiology* 8: 41.
- [11] Widjayanti T, Kusuma RR, Aini LQ, Fitriani CDA, Sektiono AW, Hadi MS & Setiawan Y. 2023. Screening of phyllospheric and endophytic bacteria as biocontrol agents of *Xanthomonas oryzae* pv. *oryzae*. *Biodiversitas* 24(4): 73-80.
- [12] da Silva JC, da Silva Júnior TAF, Soman JM, do Nascimento DM, de Melo LL, Oliveira LR, Sartori MMP & Maringoni AC. 2021. Survival of *Xanthomonas campestris* pv. *campestris* in the phyllosphere and rhizosphere of crops. *Plant and Soil* 462(389): 403-408.
- [13] Prabawati A, Susilowati A & Sugiyarto S. 2019. Phyllosphere bacteria as a candidate of biocontrol agents against *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causes bacterial blight disease. *Prosiding Seminar Nasional Masyarakat Biodiversitas Indonesia*: 256-262.

- [14] Orynbayev AT, Dzhalilov FSU & Ignatov AN. 2020. Improved efficacy of formulated bacteriophage in control of black rot caused by *Xanthomonas campestris* pv. *campestris* on cabbage seedlings. *Archives of Phytopathology and Plant Protection* 53(7-8): 379-394.
- [15] Dewi RK, Suranto AS & Wahyudi AT. 2021. Bioactive compounds of rice phyllosphere bacteria that are antagonistic toward *Xanthomonas oryzae* pv. *oryzae*. *Evolution* 25(4): 14.
- [16] Koskella B. 2020. The phyllosphere. *Current Biology* 30(19): R1143-R1146.
- [17] Holtappels D, Fortuna K, Lavigne R & Wagemans J. 2021. The future of phage biocontrol in integrated plant protection for sustainable crop production. *Current Opinion in Biotechnology* 68: 60-71.
- [18] Nurcahyanti SD, Wahyuni WS, Masnilah R & Nurdika AAH. 2021. Diversity of *Bacillus* spp. from soybean phyllosphere as potential antagonist agents for *Xanthomonas axonopodis* pv. *glycines* causal of pustule disease. *Biodiversitas* 22(11): 72-80.
- [19] Singh N & Siddiqui ZA. 2015. Effects of *Bacillus subtilis*, *Pseudomonas fluorescens* and *Aspergillus awamori* on the wilt-leaf spot disease complex of tomato. *Phytoparasitica* 43: 61-75.
- [20] Wu L, Wu H, Chen L, Yu X, Borriss R & Gao X. 2015. Difficidin and bacilysin from *Bacillus amyloliquefaciens* FZB42 have antibacterial activity against *Xanthomonas oryzae* rice pathogens. *Scientific Reports* 5(1): 12975.
- [21] Chandrasekaran M & Chun SC. 2016. Induction of defence-related enzymes in tomato (*Solanum lycopersicum*) plants treated with *Bacillus subtilis* CBR05 against *Xanthomonas campestris* pv. *vesicatoria*. *Biocontrol Science and Technology* 26(10): 1366-1378.
- [22] Jabeen R, Iftikhar T & Batool H. 2012. Isolation, characterization, preservation and pathogenicity test of *Xanthomonas oryzae* pv. *oryzae* causing BLB disease in rice. *Pakistan Journal of Botany* 44(1): 261-265.
- [23] Gregersen T. 1978. Rapid method for distinction of Gram-negative from Gram-positive bacteria. *European Journal of Applied Microbiology and Biotechnology* 5: 123-127.
- [24] Munif A, Nursalim M & Pradana AP. 2021. The potential of endophytic bacteria isolated from *Tagetes* sp. to control *Meloidogyne* spp. infection on tomato plants. *Biodiversitas* 22(6): 45-51.
- [25] Evans J, Klesius P & Shoemaker C. 2004. Starch hydrolysis testing of multiple isolates for rapid differentiation of *Streptococcus iniae*. *Bulletin-European Association of Fish Pathologists* 24(5): 231-239.
- [26] DeBritto S, Gajbar TD, Satapute P, Sundaram L, Lakshmikantha RY, Jogaiah S & Ito S-i. 2020. Isolation and characterization of nutrient dependent pyocyanin from *Pseudomonas aeruginosa* and its dye and agrochemical properties. *Scientific Reports* 10(1): 1542.
- [27] Mitrev S, Karov I, Kovacevik B & Kostadinovska E. 2014. *Pseudomonas* population causing tomato pith necrosis in the Republic of Macedonia. *Journal of Plant Pathology* 96(3): 589-592.
- [28] Syofiana RVT & Masnilah R. 2019. Eksplorasi *Bacillus* spp. pada beberapa rhizosfer gulma dan potensinya sebagai agens pengendali hayati patogen tanaman secara in vitro. *Jurnal Bioindustri* 2(1): 349-363.
- [29] Masnilah R, Astono TH & Aini LQ. 2013. Karakterisasi bakteri penyebab penyakit hawar daun edamame di Jember. *Berkala Ilmiah Pertanian* 1(1): 10-14.

- [30] Zhang H, Liu Y, Nie X, Liu L, Hua Q, Zhao G-P & Yang C. 2018. The cyanobacterial ornithine–ammonia cycle involves an arginine dihydrolase. *Nature Chemical Biology* 14(6): 575-581.
- [31] Suárez-Estrella F, Jurado M, López M, López-González J & Moreno J. 2019. Role of bacteria isolated from a plant waste-based compost producing bioactive substances in the control of bacterial spot syndrome caused by *Xanthomonas campestris* *pv.* *vesicatoria*. *Biocatalysis and Agricultural Biotechnology* 20: 101198.
- [32] Drauch V, Ibesich C, Vogl C, Hess M & Hess C. 2020. In-vitro testing of bacteriostatic and bactericidal efficacy of commercial disinfectants against *Salmonella infantis* reveals substantial differences between products and bacterial strains. *International Journal of Food Microbiology* 328: 108660.
- [33] Jonit N, Low Y & Tan G. 2016. *Xanthomonas oryzae* *pv.* *oryzae*, biochemical tests, rice (*Oryza sativa*), bacterial leaf blight (BLB) disease, Sekinchan. *Applied Environmental Microbiology* 4: 63-69.
- [34] Nawaz A, Tariq JA, Lodhi AM & Memon RM. 2020. Studies on characteristics of *Xanthomonas oryzae* isolates associated with Rice crop. *Journal of Applied Research in Plant Sciences* 1(1): 30-35.
- [35] Pradana AP, Putri D & Munif A. 2015. Eksplorasi bakteri endofit dari akar tanaman adam hawa dan potensinya sebagai agens hayati dan pemacu pertumbuhan tanaman padi. *Jurnal Fitopatologi Indonesia* 11(3): 73-78.
- [36] Sánchez-Romero MA & Casadesús J. 2020. The bacterial epigenome. *Nature Reviews Microbiology* 18(1): 7-20.
- [37] Ghsein G & Ezzeddine Z. 2022. A review of *Pseudomonas aeruginosa* metallophores: Pyoverdine, pyochelin and pseudopaline. *Biology* 11(12): 1711.
- [38] Huang H-E, Ger M-J, Yip M-K, Chen C-Y, Pandey A-K & Feng T-Y. 2004. A hypersensitive response was induced by virulent bacteria in transgenic tobacco plants overexpressing a plant ferredoxin-like protein (PFLP). *Physiological and Molecular Plant Pathology* 64(2): 103-110.
- [39] Aleklett K, Leff JW, Fierer N & Hart M. 2015. Wild plant species growing closely connected in a subalpine meadow host distinct root-associated bacterial communities. *PeerJ* 3: e804.
- [40] Santoyo G, Pacheco CH, Salmerón JH & León RH. 2017. The role of abiotic factors modulating the plant-microbe-soil interactions: toward sustainable agriculture. A review. *Spanish Journal of Agricultural Research* 15(1): 13.
- [41] Mandal M, Das S, Roy A, Rakwal R, Jones OA, Popek R, Agrawal GK & Sarkar A. 2023. Interactive relations between plants, phyllosphere microbial community, and particulate matter pollution. *Science of The Total Environment* 67(1): 164352.
- [42] Yasmin S, Hafeez FY, Mirza MS, Rasul M, Arshad HM, Zubair M & Iqbal M. 2017. Biocontrol of bacterial leaf blight of rice and profiling of secondary metabolites produced by rhizospheric *Pseudomonas aeruginosa* BRp3. *Frontiers in Microbiology* 8: 1895.
- [43] Ren H, Gu G, Longa J, Yin Q, Wu T, Song T, Zhang S, Chen Z & Dong H. 2006. Combinative effects of a bacterial type-III effector and a biocontrol bacterium on rice growth and disease resistance. *Journal of Biosciences* 31: 617-627.

- [44] Van Hop D, Hoa PTP, Quang ND, Ton PH, Ha TH, Van Hung N, Van NT, Van Hai T, Quy NTK & Dao NTA. 2014. Biological control of *Xanthomonas oryzae pv. oryzae* causing rice bacterial blight disease by *Streptomyces toxytricini* VN08-A-12, isolated from soil and leaf-litter samples in Vietnam. *Biocontrol Science* 19(3): 103-111.
- [45] Rahma H, Trisno J & Shafira S. 2023. Potential of actinobacteria as biocontrol agents to *Xanthomonas oryzae pv. oryzae* in vitro. *IOP Conference Series: Earth and Environmental Science* 532: 012040.
- [46] Pliego C, Ramos C, de Vicente A & Cazorla FM. 2011. Screening for candidate bacterial biocontrol agents against soilborne fungal plant pathogens. *Plant and Soil* 340: 505-520.
- [47] Sahu B, Singh J, Shankar G & Pradhan A. 2018. *Pseudomonas fluorescens* PGPR bacteria as well as biocontrol agent: A review. *International Journal of Chemical Studies* 6(2): 01-07.
- [48] Araya MA, Valenzuela T, Inostroza NG, Maruyama F, Jorquera MA & Acuña JJ. 2020. Isolation and characterization of cold-tolerant hyper-ACC-degrading bacteria from the rhizosphere, endosphere, and phyllosphere of antarctic vascular plants. *Microorganisms* 8(11): 1788.